Journal of Integrative Plant Biology 2006, **48** (10): 1216–1224

Identification of Novel Stress-responsive Transcription Factor Genes in Rice by cDNA Array Analysis

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Abstract

Numerous studies have shown that array of transcription factors has a role in regulating plant responses to environmental stresses. Only a small portion of them however, have been identified or characterized. More than 2 300 putative transcription factors were predicted in the rice genome and more than half of them were supported by expressed sequences. With an attempt to identify novel transcription factors involved in the stress responses, a cDNA array containing 753 putative rice transcription factors was generated to analyze the transcript profiles of these genes under drought and salinity stresses and abscisic acid treatment at seedling stage of rice. About 80% of these transcription factors showed detectable levels of transcript in seedling leaves. A total of 18 up-regulated transcription factors and 29 down-regulated transcription factors were detected with the folds of changes from 2.0 to 20.5 in at least one stress treatment. Most of these stress-responsive genes have not been reported and the expression patterns for five genes under stress conditions were further analyzed by RNA gel blot analysis. These novel stress-responsive transcription factors provide new opportunities to study the regulation of gene expression in plants under stress conditions.

Key words: abiotic stress cDNA array; *Oryza sativa*; transcription factor.

Wu CQ, Hu HH, Zeng Y, Liang DC, Xie KB, Zhang JW, Chu ZH, Xiong LZ (2006). Identification of novel stress-responsive transcription factor genes in rice by cDNA array analysis. *J Integr Plant Biol* **48**(10), 1216–1224.

doi: 10.1111/j.1672-9072.2006.00344.x; available online at www.blackwell-synergy.com, www.jipb.net

Drought and salinity are major dehydration stresses and cause adverse effects on plant growth and the productivity of crops. The physiological responses and adaptation to these stresses are initially resulted from the changes of gene expression triggered by stresses. Expression of eukaryotic genes often relies on specific transcription factors (TFs) that bind to or modulate DNA structure in the regulatory region of genes, which in turn affects the activity of RNA polymerases for initiation of transcription. Recently, numerous studies have shown that TFs play important roles in regulating the responses to various stresses in plants and some of them have been shown to be essential for stress tolerance. In *Arabidopsis*, transcription factors belonging to various subfamilies such as DREB1A and DREB2A of AP2 family (Liu et al. 1998), AREB1, AREB2, and AREB3 of bZIP family (Uno et al. 2000), Atmyb2, CpMYB10 and BOS1 of MYB family (Urao et al. 1993; Mengiste et al. 2003; Villalobos et al. 2004), RD26 , ANAC019, ANAC055, and ANAC072 of NAC family (Fujita et al. 2004; Tran et al. 2004), and zinc finger proteins AZF1, AZF2, AZF3, STZ and ZPT2-3 (Sugano et al. 2003; Sakamoto et al. 2004) have been implicated in plant stress responses.

In rice, only few transcription factors have been reported to be involved in abiotic stress responses. Five cDNAs of DREB homologs (*OsDREB1A*, *OsDREB1B*, *OsDREB1C*, *OsDREB1D*, and *OsDREB2A*) have been isolated in rice

Received 18 May 2005 Accepted 10 Aug. 2005

Supported by the National Natural Science Foundation of China (30270713) and the State Key Basic Research and Development Plan of China (2002CB108804).

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(Dubouzet et al. 2003). Among them, *OsDREB1A* and *OsDREB1B* were induced by cold, whereas *OsDREB2A* was induced by dehydration and high-salt stresses. Overexpression of *OsDREB1A*, a functional analog to *DREB1A* in *Arabidopsis*, showed potential usefulness in producing transgenic plants that were tolerant to drought, high-salinity, and/or cold stresses (Dubouzet et al. 2003). The transcript of *OsDREBL*, containing an AP2 DNA binding domain, accumulated within 30 min in response to low temperature, but not in response to abscisic acid (ABA), NaCl and dehydration treatments (Chen et al. 2003). Further study demonstrated that *OsDREBL* did not bind effectively to the C-repeat/dehydration responsive element (CRT/DRE), suggesting that *OsDREBL* may function as a transcription factor in the cold-stress response and is independent of the DREB-mediated pathway (Chen et al. 2003). *OSISAP1*, encoding a zinc-finger protein in rice, was induced by various stresses including cold, desiccation, salt, submergence, heavy metals, injury and ABA treatment, and overexpression of *OSISAP1* in tobacco led to enhanced tolerance to cold, dehydration, and salt stresses at the germination and seedling stages (Mukhopadhyay et al. 2004).

The release of the genome sequence of *Arabidopsis* (Arabidopsis Genome Initiative, 2000) and rice (Feng et al. 2002; Goff et al. 2002; Sasaki et al. 2002; Yu et al. 2002) and increasingly matured cDNA microarray or DNA chip technologies have provided new opportunities to decipher the information hidden behind the vast number of nucleotide sequences. In addition to the genomic scale of expression profiling, studying the expression pattern of a subset of functionally important genes such as TFs can provide more intensive information on gene expression and regulation (Czechowski et al. 2004). A recent study on the expression profiles of 402 *Arabidopsis* transcription factor genes showed that 20% of the transcription factor genes were responsive to various stress treatments (Chen et al. 2002). It is obvious that an analysis of a more complete collection of transcription factor genes will provide more opportunities in identification of new transcription factors involved in the response to stresses.

Based on the prediction of putative transcription factors in rice genomes, this study aimed to isolate and identify novel stress responsive transcription factor genes. A DNA array containing 753 rice TFs was generated and used to analyze the transcript level of these genes in the rice seedlings treated with drought, salt, or ABA. A total of 45 putative transcription factors showing expression changes in at least one of these stresses were identified.

Results

Genomic analysis of putative TFs in rice

By key word searching, 4 023 putative TF entries were obtained including 1 706 entries from EST or full-length cDNA databases and 2 317 entries from the annotation of rice genomic sequences. Redundant entries were removed based on the chromosomal locations. A total of 2 344 non-redundant putative TFs were estimated in rice genomes. However, only 1 313 (56%) of them were currently supported with EST or full-length cDNAs. The percentages of TF genes in different families (Table 1) were generally similar to that in *Arabidopsis* (Riechmann et al. 2000). However, the family of zinc finger with several subfamilies that are thought to have evolved independently (Berg and Shi 1996), accounts for approximately 34% of all TFs and is apparently more than the number in *Arabidopsis* (<22%). The other three largest families of transcription factors are AP2/EREBP (APETALA2/ethylene responsive element binding protein), MYB and bHLH (basic helix-loop-helix), each accounting for about 9% of the total entries both in rice and *Arabidopsis*.

Isolation of gene-specific fragments of transcription factors

From the 423 putative TF clones identified in our cDNA library, 415 were amplified using gene-specific primers. Another 400 pairs of primers were designed to amplify TF-specific fragments and specific bands with expected sizes (Figure 1) were generated for 352 (88%) genes using the first strand cDNA as template. Sequencing analysis suggested that 338 of them had expected sequences and were then used for making the DNA array. Together with negative (plasmid of pUC18) and positive (rice *Actin1*) control, 755 fragments were used for DNA array hybridization. The 753 putative TFs included 118 AP2/EREBP

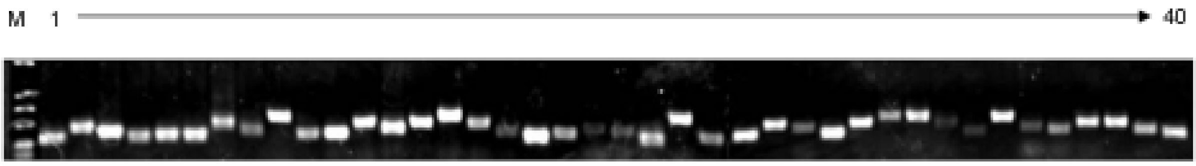

Figure 1. The efficiency of RT-PCR amplification of gene-specific fragments for DNA array.

M, 2 kb DNA ladder; 1–40, PCR fragments for putative transcription factors.

Table 1. Number of predicted transcription factor genes in the rice genome

Gene family[a]	No. genes	%
AP2/ERF	188	8.0
bHLH	160	6.8
MYB	215	9.2
C2H2 (Zn)	197	8.4
NAC	135	5.8
HB	69	2.9
MADS	78	3.3
bZIP	72	3.1
WRKY (Zn)	112	4.8
GARP : G2-LIKE	49	2.1
GARP : ARR-B	30	1.3
C2C2 : DOF	33	1.4
C2C2 : CO-like	34	1.5
C2C2 : GATA	30	1.3
C2C2 : YABBY	6	0.3
CCAAT type	43	1.8
GRAS	52	2.2
TRIHELIX	25	1.1
HSF	28	1.2
TCP	25	1.1
ARF	32	1.4
C3H	334	14.2
SBP	21	0.9
ABI3/VP1	52	2.2
TUB	13	0.6
E2F/DF	8	0.3
CPP(Zn)	12	0.5
Alfin-like	50	2.1
EIL	8	0.3
Aux/IAA	37	1.6
HMG-box	14	0.6
ARID	5	0.2
JUMONJI	17	0.7
Others	160	6.8
Total	2 344	100

[a]Genes are classified based on sequence similarity to known transcription factors.

genes, 121 MYB genes, 34 bZIP genes, 33 MADS genes, 39 bHLH genes, 32 homeobox (HB) genes, 302 zinc finger genes consisting of different subgroups, 7 NAC genes, 5 IAA/AXR genes and 32 genes without classification (Table 2).

Expression level of selected transcription factor genes in seedling leaves

The expression level of the transcription factor genes in normal growing seedlings was estimated based on the six independent biological samples that were served as a control for stress experiments. Six hundred and one out of the 753 transcription factor genes showed significant ($P < 0.05$) higher signal intensity than the negative control (background), suggesting 79.8% transcription factor genes were expressed in seedling leaves. These expressed genes were distributed among all selected groups (Table 2), including 95 AP2/EREBP genes (80.5% of this family), 96 MYB genes (79.3%), 28 bZIP genes (82.4%), 27 MADS genes (81.8%), 30 bHLH genes (76.9%), 27 HB genes (84.5%), 139 C2H2 (ZF) (75.1%) genes, and five NAC genes (71.4%). These results demonstrated that a majority of the transcription factor genes in this study were expressed in seedling leaves.

Table 2. Number of transcription factors expressed in seedling leaves

Gene family	Gene arrayed		Gene expressed	
	No.	%	No.	%
AP2/ERF	118	15.7	95	80.5
bHLH	39	5.2	30	76.9
MYB	121	16.1	96	79.3
C2H2 (Zn)	185	24.6	140	75.7
NAC	7	0.9	5	71.4
HB	32	4.2	27	87.5
MADS	33	4.3	27	81.8
bZIP	34	4.5	28	82.4
WRKY (Zn)	40	5.3	34	82.5
C2C2 : DOF	10	1.3	8	80.0
C2C2 : CO-like	18	2.4	14	77.8
C2C2 : GATA	12	1.6	10	83.3
C2C2 : YABBY	5	0.7	4	80.0
HSF	15	2.1	12	80.0
ARF	8	1.1	6	75.0
C3H	30	4.0	24	81.5
SBP	7	0.9	6	82.3
CPP (Zn)	5	0.7	4	84.1
Aux/IAA	5	0.7	4	79.0
Others	32	4.2	27	83.7
Total	753	100	601	79.8

Stress-responsive transcription factors in rice seedlings

The signal intensity of the 753 transcription factor genes was quantified and normalized based on the signal intensity of the positive control between treatment and control. A total of 18 TF genes showed increased expression and 29 TF genes showed decreased expression in at least one stress treatment (Figure 2). The fold of changes ranged from 2.0 to 20.5 (Table 3). Eleven TFs were responsive to multiple stresses (Figure 3). The expressions of two genes, *TF01L12* (a MADS-box gene)

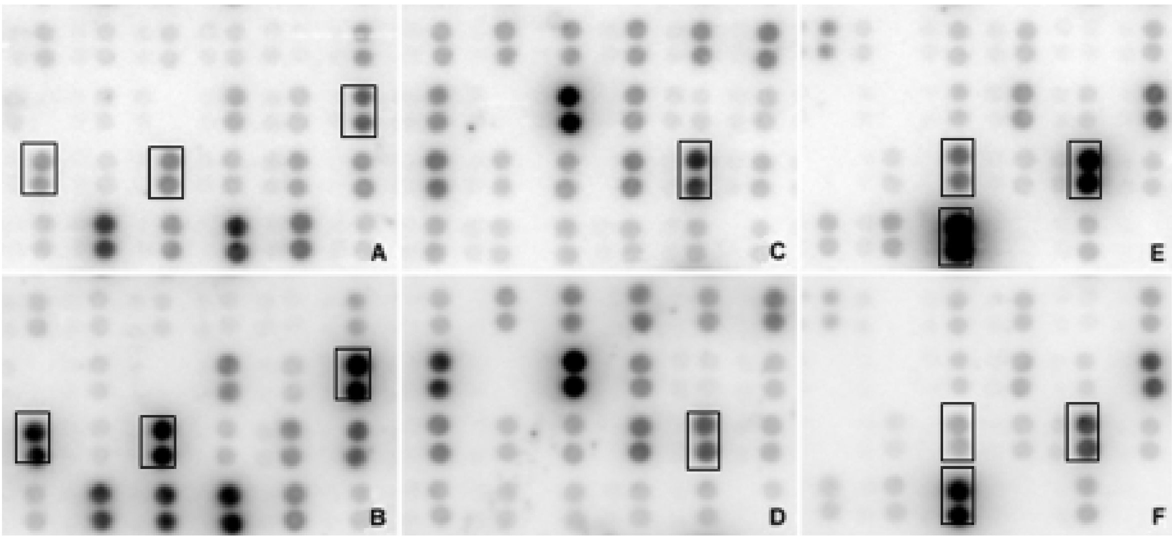

Figure 2. Identification of stress-responsive TFs by DNA array analysis.

Two independent samples for each stress treatment and control were used for array hybridization at different times. A part of the array was shown for one repeat: A–B, dehydration versus control; C–D, ABA versus control; E–F, salt stress versus control. The boxes indicate differentially expressed clones between stress treatment and controls.

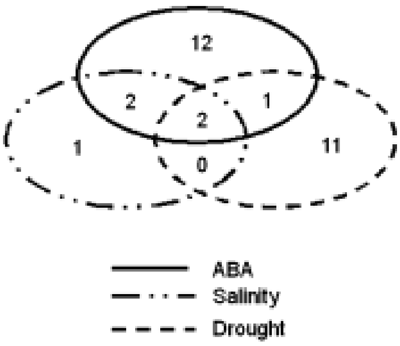

Figure 3. Diagram of the number of TF genes responsive to different stresses.

and *TF01M13* (a C2C2 type zinc finger gene), were decreased in all the three treatments. Two genes: *TF03D07*, and *TF01K23*, were down regulated by ABA and salinity, but not by drought. Five TF genes were induced by ABA and salinity (Table 3). Only one TF was down regulated by both ABA and drought. All the TFs showing changed transcript levels under stress conditions in this study have not been reported in rice.

Confirmation of the stress responsive TFs by RNA gel blot analysis

To validate the stress-responsive TFs identified from array hybridization, five TF genes representing low (2–3-fold), mediate (4–6-fold) and high level (>8-fold) differential expressions were chosen for RNA gel blot analysis. As shown in Figure 4, all the selected genes showed stress-induced or suppressed expressions and were in agreement with the array results. *TF01D04*, a homology of *rough sheath 2* gene showing a 2.2-fold reduction by ABA treatment in the array hybridization, exhibited a gradually suppressed expression pattern from 30 min to 6 h after ABA stress (Figure 4A). The transcript of *TF03G07*, encoding an XH/XS domain-containing protein with a 10.2-fold induction by drought in array analysis was increased gradually when relative water content (RWC) in leaves was decreased (Figure 4B). The expression level of *TF03D07* — a zinc finger gene with a 5.3-fold suppression by salt stress in the array analysis — dropped sharply 1 d after the salinity stress (Figure 4C). *TF01C06* (Figure. 4 D–F) and *TF01I01* (Figure 4 G–I), two AN1-like zinc finger genes showed similar expression patterns under three different treatments and their transcripts steadily increased after ABA and salt treatment, but dropped in the late stage of drought stress.

Discussion

Previous reports suggested that there are at least 1 864 transcription factors in the *Arabidopsis* genome (Riechmann et al. 2000; Jiao et al. 2003;). A complete database search suggested

Table 3. Transcription factors showing a greater than twofold increase or decrease in expression following stress treatments

Array ID	Accession[a]	Expression change	Stress	Fold change[b]	Annotation of the highest homolog	E value
TF01C06	AK060008	Up	D, A, S	3.1, 3.7, 8.2	AN1-like zinc finger family protein	1E-52
TF01C08	AK061661	Up	S	4.4	Putative transcription factor BTF3 mRNA (*Oryza sativa*)	6E-66
TF01C15	AK105422	Down	D	2.0	Putative transcription factor	1E-57
TF01D04	AK068492	Down	A	2.2	Transcription factor (rough sheath 2 like protein)	2E-63
TF01D11	AK103787	Up	A	8.4	KH domain-containing protein/zinc finger (CCCH type)	4E-41
TF01E22	AK067419	Up	S	6.8	RNA recognition motif (RRM)-containing protein	7E-93
TF01F02	AK061433	Up	S	5.5	Heat shock factor protein 4 (HSF4)	2E-41
TF01I01	AK104605	Down	D, A, S	4.0, 2.8, 8.6	Zinc finger (AN1-like) family protein	1E-52
TF01L06	AK071272	Down	S	3.7	ZIP4 (*Thlaspi caerulescens*)	2E-83
TF01N01	AK072361	Up	A, S	5.4, 4.2	P-type R2R3 Myb protein gene	2E-41
TF01N02	AK068392	Up	A, S	6.4, 3.7	No apical meristem (NAM) family protein	2E-97
TF01N09	AK101729	Down	A	8.8	ARF GAP-like zinc finger-containing protein (ZIGA4)	7E-81
TF01P22	AK071713	Down	D	8.3	Putative transcription factor	1E-119
TF02A14	AK108510	Up	D	2.0	Putative transcription factor	6E-45
TF02A15	AK072440	Up	S	6.6	Putative transcriptional coactivator	4E-22
TF02A20	AK065504	Down	A	20.5	Putative transcription factor	1E-72
TF02E13	AK102951	Up	A	8.3	Basic helix-loop-helix (bHLH) family protein	2E-56
TF02E14	AK103400	Up	D, A	7.8, 9.5	Remorin family protein	2E-24
TF02E23	AK102093	Down	A	12.8	WRKY family transcription factor	2E-14
TF02I14	AK107867	Down	A	10.4	Putative transcription factor	1E-107
TF02I19	AK072942	Down	D	2.8	Zinc finger (C2H2 type) family protein	4E-25
TF02I20	AK100322	Down	D, A	2.3, 8.1	Transcriptional factor B3 family protein	0
TF02M08	AK072130	Down	D	4.0	Putative transcription factor	2E-43
TF02M13	AK068990	Down	A	4.6	Expressed protein	1E-126
TF02N21	AK107637	Down	A	5.7	Homeobox protein knotted-1 like 1 (KNAT1)	5E-59
TF02O04	AK099864	Down	A	5.0	Zinc finger (B-box type) family protein	3E-26
TF02O23	AK105957	Up	S	6.0	Zinc finger (B-box type) family protein	9E-34
TF02P13	AK101620	Down	A	3.3	bZIP family transcription factor	1E-101
TF02P23	AK102194	Up	A	4.7	TUA2-like protein	0
TF03B08	AK065522	Down	A	2.5	Putative transcription factor	3E-62
TF03B20	AK104073	Up	A, S	4.7, 5.4	myb family transcription factor	2E-36
TF03C13	AK066252	Down	D	17.4	WRKY transcription factor/WRKY1 (*Avena sativa*)	4E-32
TF03D07	AK059839	Down	A, S	6.3, 5.3	Zinc finger (C2H2 type) family protein (ZAT10)	9E-27
TF03D15	AK100276	Down	D	2.0	*Arabidopsis thaliana* unknown protein (At1g57680)	9E-60
TF03E22	AK070466	Down	A	2.6	bZIP transcription factor family protein	1E-60
TF03E23	AK068187	Down	D	6.0	Similar to nuclear receptor binding factor-1 (NRBF-1)	9E-51
TF03F01	AK068181	Down	D	7.9	Putative transcription factor	5E-90
TF03F06	AK058671	Up	D	2.2	Putative RNA Binding Protein 45	7E-57
TF03G07	AK063522	Up	D	10.2	XH/XS domain-containing protein	1E-118
TF03G22	AK068281	Down	D	5.6	RNA binding protein (*Arabidopsis)*	1E-139
TF03H23	AK109360	Up	D	10.6	AP2 domain-containing protein RAP2.3	9E-28
TF01K23	AK101377	Down	A, S	12.0, 3.7	Putative transcription factor	0
TF01L12	AK072683	Down	D, A, S	3.8, 13.5, 8.4	*Oryza sativa* MADS15 protein mRNA	9E-56
TF01M13	AK109477	Down	D, A, S	4.0, 8.9, 6.9	Zinc finger (GATA type) family protein	7E-34

[a]Accession numbers from GenBank (http://www.ncbi.nlm.nih.gov/) were used for the putative TF genes.

[b]Average values based on two independent experiments.

A, abscisic acid treatment; D, drought stress; S, salinity stress.

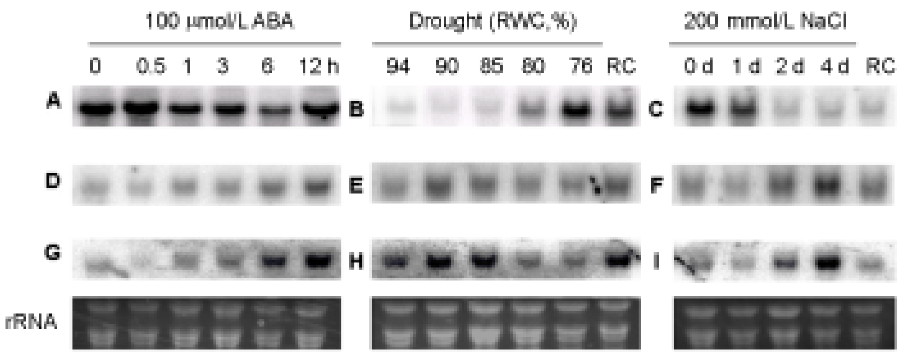

Figure 4. RNA gel blotting analysis for five TF genes, *TF01D04* **(A)**, *TF03G07* **(B)**, *TF03D07* **(C)**, *TF01C06* **(D–E)** and *TF01I01* **(G–I)** under stress conditions.

The numbers in drought treatment indicate relative water content (RWC, about 94% for CK) in leaves; RC, recovery for 1 d by re-watering after stresses. The total RNA loaded for blotting was indicated in the bottom row.

that about 2 300 putative transcription factor genes exist in the rice genome. This is not surprising considering the larger genome size of rice than *Arabidopsis*. The difference in the number of TFs in different families, however, is not always similar between these two species. For example, zinc finger proteins account for 34% of total TFs in rice, which is surprisingly more than that in *Arabidopsis* (22%). Zinc finger family can be grouped into several subfamilies based on the structural features of zinc-coordinated motif. These subfamilies include plant-specific (Eulgem et al. 2000) and DOF proteins (Yanagisawa and Schmidt 1999), GATA-type zinc finger proteins (Jensen et al. 1998), and others shown in Table 1. The number of genes in these subfamilies also varied between rice and *Arabidopsis*.

The prediction and classification of putative TFs in the rice genome were based on sequence comparisons of rice cDNAs or predicted genes with known TFs. Therefore, the total number of TFs could be overestimated due to the marginal homology for some sequences or pseudogenes predicted from genomic sequences, and the classification for some individual genes may not functionally right. For example, in addition to the MYB proteins, putative transcription factors characterized by a divergent MYB domain existed in the rice genome. These genes consist of a divergent group and are often referred to as “MYB related”. For simplicity, all of the MYB-related proteins in this study were put into the MYB family.

With an intension to isolate novel TFs responsive to abiotic stresses, a DNA array containing 753 predicted rice TFs (not including published TF genes) were hybridized with cDNA probes from drought, salt and ABA-treated seedling leaves. A total of 45 genes were identified to be responsive to at least one stress treatment. The percentage (6%) of stress-responsive TF in this study is much lower than that (20%) in *Aarabidopsis* (Chen et al. 2002), which might mainly be due to three reasons. First, all reported stress-responsive rice TFs were not included in this analysis. Second, the number of stress treatments used in this study was much fewer than that in *Arabidopsis* (Chen et al. 2002). Third, the threshold for claiming expression changes in this study was rather strict (2-fold induction or suppression) considering the sensitivity of the isotope probe is generally lower than the fluorescence probe, thus some genes with low levels of induction or suppression might be missed. Nevertheless, five genes with different levels of expression changes were confirmed by RNA gel blotting analysis, suggesting that most, if not all TFs identified in this study were indeed responsive to the stresses specified in Table 3. Besides some TFs belonging to widely accepted stress responsive TF families (such as AP2/EREBP, MYB and NAC), more than half of these stress-responsive TFs (belonging to families such as homeobox, ARF, and C3H zinc finger), have not been reported to be involved in abiotic stresses. Eleven TFs were responsive to more than one stress, suggesting a cross-talk of different stress responses as suggested in *Arabidopsis* (Shinozaki and Yamaguchi-Shinozaki 2000). Although only a partial collection of TFs in the rice genome were profiled for their expressions under a few major abiotic stresses, all the stress-responsive TF genes identified in this study have not been reported and these genes may provide new opportunities to understand the specific transcriptional regulation in the response to different abiotic stresses in rice.

Materials and Methods

Plant material and stress treatment

An upland rice IRAT109 (*Oryza sativa* L. ssp. *japonica*) showing strong tolerance to drought (Yue et al. 2006) was used in this study. Four-leaf-old healthy seedlings growing in homogenized soil in a greenhouse were subjected to stress treatment. Drought stress was applied by stopping watering, and relative water content (RWC) in leaves was measured every day at noon. Leaf samples from early (RWC at 90%), intermediate (RWC at 85%) and late (RWC at 76%) stages of drought stress were harvested. For salinity stress, NaCl was added with a final concentration of 200 mmol/L and leaf tissue was sampled daily at noon for 4 d. Seedling leaves were sprayed with 100 μmol/L ABA treatment and harvested at 30 min, 1 h, 3 h, 6 h and 12 h respectively. Control samples were harvested at the time points parallel to the corresponding stress treatment.

Database mining for transcription factors in rice genome

A comprehensive search was performed in the annotation database of rice genome (TIGR) and the full-length cDNA database (KOME) using key words including “transcription factor” and the subfamily names of transcription factors as used in *Arabidop*sis (Riechmann et al. 2000). A similar search was performed to find published rice cDNAs encoding putative TFs in GenBank. In addition, putative TFs were identified from a cDNA library containing more than 20 000 unique ESTs (Chu et al. 2003) by BLASTX search against the protein database. The classification of putative rice TFs was based on the annotation of cDNAs or predicted genes that was derived from the annotation of the hit with the highest sequence identity using BLASTN, BLASTX or BLASTP programs (Altschul et al. 1997) with score values more than 100. Putative TFs without distinct classification and TFs belonging to very small gene families were classified as “others”. The location of rice transcription factor on chromosomes was determined by the physically chromosome-localized rice BAC sequences (TIGR database), to which transcription factor sequences were mapped using BLASTN program. A detailed list of these putative transcription factor genes (with information of classification, annotation and chromosomal localization) can be found at http://www.Ricefgchina.org/tf/.

Isolation of gene-specific fragments for DNA array

To isolate TF fragments for making a DNA array, primers with an average length of 18–22 bp were designed to produce fragments of 300 to 800 bp in length, with sequences specific for the selected TF genes. The score value was lower than 50 for the hit other than itself in the BLASTN search against genome sequences. The rice putative transcription factor genes not published but supported with full-length cDNAs or ESTs were selected with priority for amplification. From a cDNA library with more than 20 000 unique cDNAs (Chu et al. 2003), about 423 putative TFs were identified and these clones were also used as templates for amplification. Other TF fragments were generated by PCR using the first strand cDNA of drought-stressed seedling leaves from the upland rice IRAT109. For comparison of hybridization signals between blots, rice *Actin1* gene was amplified and used as a positive control. The PCR reaction was performed following standard PCR protocol in a volume of 100 mL containing 0.1 mmol/L each of primer, 20 ng of template and 2.5 U *Taq*. The PCR products (5 mL of each reaction) were separated on 1.2% agarose gel to check the amplification quality and the size of bands. Qualified (sufficient amount of amplification and expected size of band) PCR products were purified for sequencing. Sequencing was performed on ABI3700 System (Applied Biosystems, Foster City, CA). Only sequence-confirmed clones were used to make the array.

DNA array analysis

The PCR products were purified by ethanol precipitation and dissolved in water with a concentration of about 100 ng/mL. The PCR products were arrayed onto the Hybond-N^+ membrane (Amersham Piscataway, NJ), two dots for each sample, with Biomek 2000 laboratory automation workstation (Beckman, Fullerton, CA). DNA-printed membrane was laid sequentially on three filter papers, each for 5 min, saturated with solutions I (0.5 mol/L NaOH and 1.5 mol/L NaCl), II (0.5 mol/L Tris-HCl, pH 7.5) and III (2× SSC, 0.1% SDS) respectively. After air-drying, the membrane was baked in a vacuum oven at 80 ºC for 2 h.

Total RNA was isolated from treated and untreated (control) rice leaves at 4-leaf stage with TRIZOL reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. RNA samples from the time courses of treatment or control were equally mixed for probe labeling. The reverse transcription reaction was performed in a volume of 60 µL containing 15 µg total RNA, 1.5 µg Oligo$(dT)_{15}$ primer (Promega, Madison, WI), 600 U of Superscripts II reverse transcriptase (Inventrigen), 500 µmol/L each of dATP, dGTP and dTTP, and 50 µCi ^{32}P-dCTP (3 000 Ci/mmol). After incubation at 42 ºC for 1 h, the reaction was stopped and RNA was degraded by adding 5 µL of 0.5 mol/L NaOH and 5 µL of 100 mmol/L EDTA and incubated at 70 ºC for 10 min. The probe was purified using Sephadex G-50 column. Membranes were prehybridized for 1 h and hybridized with a probe overnight, using PerfectHYB Plus buffer (Sigma, St. Luis, MO) at 65 ºC. After hybridization, the membranes were washed sequentially with 2×SSC and 0.1% SDS, 1×SSC and 0.1% SDS, and 0.5×SSC for 20 min each wash at 65 ºC.

Quantification of hybridization signals was conducted in PhosphorImager SI (Molecular Dynamics) using the program ArrayGauge Version 1.0 (FUJI Photo Film Co. LTD). Hybridization signals of TF genes were normalized to the signal of *Actin1* gene in each blot. Differentially expressed genes were determined based on three criteria: (i) at least two folds of signal intensity difference between control and stress treatments (such a threshold of difference was visually distinguishable); (ii) repeated in two experiments using independent biological samples; and (iii) weak background around the putative differential expressed clones.

RNA gel blot analysis

Total RNA (15 μg) was resolved in 1.2% denaturing agarose gel containing 2% formaldehyde and transferred to Hybond-N^+ membranes (Amersham). Membranes were cross-linked by UV light. Blots were prehybridized for 1 h and hybridized with ^{32}P-dCTP-labelled gene-specific DNA probe for overnight using PerfectHYB Plus buffer (Sigma) at 65 ºC. Blots were washed three times (twice each with 2×SSC /0.1% SDS for 20 min and once with 0.5×SSC /0.1% SDS for 20 min) at 65 ºC. The blots were briefly air-dried and then subjected to radiography.

References

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W et al. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.

Arabidopsis Genome Initiative (2000). Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature* **408**, 796–815.

Berg JM, Shi Y (1996). The galvanization of biology: a growing appreciation for the roles of zinc. *Science* **271**, 1081–1085.

Chen JQ, Dong Y, Wang YJ, Liu Q, Zhang JS, Chen SY (2003). An AP2/EREBP-type transcription-factor gene from rice is cold-inducible and encodes a nuclear-localized protein. *Theor Appl Genet* **107**, 972–979.

Chen W, Provart NJ, Glazebrook J, Katagiri F, Chang HS, Eulgem T et al. (2002). Expression profile matrix of *Arabidopsis* transcription factor genes suggests their putative functions in response to environmental stresses. *Plant Cell* **14**, 559–574.

Chu ZH, Peng KM, Zhang LD, Zhou B, Wei J, Wang SP (2003). Construction and characterization of a normalized whole-life-cycle cDNA library of rice. *Chin Sci Bull*, **48**, 229–235.

Czechowski T, Bari RP, Stitt M, Scheible WR, Udvardi MK (2004). Real-time RT-PCR profiling of over 1 400 *Arabidopsis* transcription factors: unprecedented sensitivity reveals novel root- and shoot-specific genes. *Plant J* **38**, 366–379.

Dubouzet JG, Sakuma Y, Ito Y, Kasuga M, Dubouzet EG, Miura S et al. (2003). *OsDREB* genes in rice, *Oryza sativa* L., encode transcription activators that function in drought-, high-salt- and cold-responsive gene expression. *Plant J* **33**, 751–763.

Eulgem T, Rushton PJ, Robatzek S, Somssich IE (2000). The WRKY superfamily of plant transcription factors. *Trends Plant Sci* **5**, 199–206.

Feng Q, Zhang Y, Hao P, Wang S, Fu G, Huang Y et al. (2002). Sequence and analysis of rice chromosome 4. *Nature* **420**, 316–320.

Fujita M, Fujita Y, Maruyama K, Seki M, Hiratsu K, Ohme-Takagi M et al. (2004). A dehydration-induced NAC protein, RD26, is involved in a novel ABA-dependent stress-signaling pathway. *Plant J* **39**, 863–876.

Goff SA, Ricke D, Lan TH, Presting G, Wang R, Dunn M et al. (2002). A draft sequence of the rice genome (*Oryza sativa* L. ssp. *japonica*). *Science* **296**, 92–100.

Jensen RB, Jensen KL, Jespersen HM, Skriver K (1998). Widespread occurrence of a highly conserved RING-H2 zinc finger motif in the model plant *Arabidopsis thaliana*. *FEBS Lett* **436**, 283–287.

Jiao Y, Yang H, Ma L, Sun N, Yu H, Liu T et al. (2003). A genome-wide analysis of blue-light regulation of *Arabidopsis* transcription factor gene expression during seedling development. *Plant Physiol* **133**, 1480–1493.

Liu Q, Kasuga M, Sakuma Y, Abe H, Miura S, Yamaguchi-Shinozaki K et al. (1998). Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in *Arabidopsis*. *Plant Cell* **10**, 1391–1406.

Mengiste T, Chen X, Salmeron J, Dietrich R (2003). The BOTRYTIS SUSCEPTIBLE1 gene encodes an R2R3MYB transcription factor protein that is required for biotic and abiotic stress responses in *Arabidopsis*. *Plant Cell* **15**, 2551–2565.

Mukhopadhyay A, Vij S, Tyagi AK (2004). Overexpression of a zinc-finger protein gene from rice confers tolerance to cold, dehydration, and salt stress in transgenic tobacco. *Proc Natl Acad Sci USA* **101**, 6309–6314.

Riechmann JL, Heard J, Martin G, Reuber L, Jiang C, Keddie J et al. (2000). *Arabidopsis* transcription factors: Genome-wide comparative analysis among eukaryotes. *Science* **290**, 2105–2110.

Sakamoto H, Maruyama K, Sakuma Y, Meshi T, Iwabuchi M, Shinozaki K et al. (2004). *Arabidopsis* Cys2/His2-type zinc-finger proteins function as transcription repressors under drought, cold, and high-salinity stress conditions. *Plant Physiol* **136**, 2734–2746.

Sasaki T, Matsumoto T, Yamamoto K, Sakata K, Baba T, Katayose Y et al. (2002). The genome sequence and structure of rice chromosome 1. *Nature* **420**, 312–316.

Shinozaki K, Yamaguchi-Shinozaki K (2000). Molecular responses to dehydration and low temperature: Differences and cross-talk between two stress signaling pathways. *Curr Opin Plant Biol* **3**, 217–223.

Sugano S, Kaminaka H, Rybka Z, Catala R, Salinas J, Matsui K et al. (2003). Stress-responsive zinc finger gene *ZPT2-3* plays a role in drought tolerance in *petunia*. *Plant J* **36**, 830–841.

Tran LS, Nakashima K, Sakuma Y, Simpson SD, Fujita Y, Maruyama K et al. (2004). Isolation and functional analysis of Arabidopsis stress-inducible NAC transcription factors that bind to a drought-responsive *cis*-element in the early responsive to dehydration stress 1 promoter. *Plant Cell* **16**, 2481–2498.

Uno Y, Furihata T, Abe H, Yoshida R, Shinozaki K, Yamaguchi-Shinozaki K (2000). Arabidopsis basic leucine zipper transcription factors involved in an abscisic acid-dependent signal transduction pathway under drought and high-salinity conditions. *Proc Natl Acad Sci USA* **97**, 11 632–11 637.

Urao T, Yamaguchi-Shinozaki K, Urao S, Shinozaki K (1993). An *Arabidopsis* myb homolog is induced by dehydration stress and its gene product binds to the conserved MYB recognition sequence. *Plant Cell* **5**, 1529–1539.

Villalobos M A, Bartels D, Iturriaga G (2004). Stress tolerance and glucose insensitive phenotypes in *Arabidopsis* overexpressing the CpMYB10 transcription factor gene. *Plant Physiol* **135**, 309–324.

Yanagisawa S, Schmidt RJ (1999). Diversity and similarity among recognition sequences of Dof transcription factors. *Plant J* **17**, 209–214.

Yu J, Hu S, Wang J, Wong GK, Li S, Liu B et al. (2002). A draft sequence of the rice genome (*Oryza sativa* L. ssp. *indica*). *Science* **296**, 79–92.

Yue B, Xue W, Xiong L, Yu X, Luo L, Cui K et al. (2006). Genetic basis of drought resistance at reproductive stage in rice: Separation of drought tolerance from drought avoidance. *Genetics* **172,** 1213–1228.

(Managing editor: Li-Hui Zhao)